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Determination of Haemoglobin and Prothrombin Complex in Whole Blood using Optothermal Spectrometry

By *P. Masson*

Department of Clinical Chemistry, University Hospital, Lund, Sweden

H. Elwing, I. Lundström

Laboratory of Applied Physics, Linköping Institute of Technology, Linköping, Sweden

J. O. Karlsson

Department of Pharmacology, Linköping University Medical Hospital, Linköping, Sweden and

P. Helander

AB Varilab, Huddinge, Sweden

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Summary: Optothermal spectrometry measures the thermal energy produced as a result of absorption by molecules at a given modulation frequency and wavelength. Depending on the modulation frequency used, analysis can be performed in very thin layers (50–150 micrometers). A major advantage of optothermal spectrometry is that it is not very sensitive to light scattering. Haemoglobin in whole blood was measured without any reagent at 16 Hz and 2 Hz frequencies. The precisions (within-series, within-day and between-day) were acceptable, and comparisons with reference methods were excellent. As opposed to the reference methods, optothermal spectrometric determinations were not affected by lipaemia. When measured continuously, the signal for haemoglobin increased due to the packing of erythrocytes towards the light source. By converting soluble fibrinogen into insoluble fibrin, the erythrocyte packing could either be inhibited or stopped, and this process could be monitored by assessing the change in the rate of signal increase for haemoglobin. This principle was utilized to analyse the prothrombin complex; the method was found to have acceptable precision and to be comparable to a routine method.

Introduction

The most commonly used optical methods for quantitative analyses are transmission and reflection spectrometry where the concentration of an analyte is measured either on the basis of its transmission or reflection properties. One drawback with these methods is that light-scattering in the sample strongly interferes with the measurement. This problem can be circumvented if the amount of light absorbed directly by the sample is measured. This can be achieved by detecting the temperature rise due to absorbed light and this principle forms the basis of optothermal spectrometry. Light impulses at a particular modulation frequency and a chosen wavelength are incident on a sample through a thin sapphire window (1, 2) as shown in figure 1. Each pulse causes a small temperature increase in the sample and this is transmitted to the sapphire. The subsequent thermal expansion of the sapphire is detected by a piezoelectric crystal and converted into an electrical signal. This signal has the same frequency as the modulation frequency of the light pulses and an amplitude depending upon

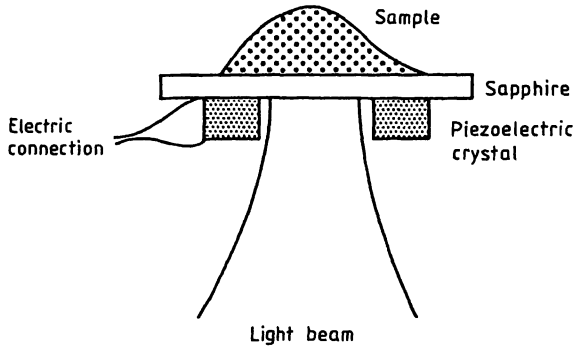

Fig. 1. Cross sectional view of the optothermal detector.

the amount of light absorbed close to the sapphire (3). Light scattering changes the distribution of light in the sample and the amount of light close to the sapphire. However this effect is much less severe than that observed with other techniques, and can be compensated for by the calibration.

The thickness of the analysed layer depends on the speed at which the heat generated propagates from the sample to the sapphire. This distance is denoted as the thermal diffusion length (3) and depends inversely on the square root of the modulation frequency being used, a high frequency is required for analysis in a thin layer and vice versa. The present study was conducted either at 2 or 16 Hz frequency, where the thermal diffusion lengths in water are 149 and 52 micrometers, respectively. Hence optothermal spectrometry is well suited to analyse strongly absorbing samples such as whole blood, as well as samples where light scattering is a practical problem. The fact that only a small part of the sample is analysed (the sample is usually much thicker than the thermal diffusion length) gives an extra dimension for this technique. For whole blood, the number of erythrocytes within the analysis layer increases with time due to sedimentation, and this effect can be monitored (4). The sedimentation of erythrocytes has also been measured by a related technique called photoacoustic spectroscopy, where a microphone is used to measure pressure variations in a closed cell. Due to the instrumental design, the sedimentation is measured as a decreasing signal (5).

The use of optothermal spectrometry to analyse haemoglobin and the prothrombin complex using whole blood is presented here.

(I) Determination of Haemoglobin

Materials and Methods

Blood collected in EDTA for routine haematology was used. The optothermal spectrometer, constructed by AB Varilab, operates at two frequencies (16 and 2 Hz) by means of a mechanical chopper as shown in figure 2. A 20 Watt halogen lamp was used as the light source and a band pass filter (Schott, West Germany, filter BG 18, 540 ± 40 nm) was used for the determination of haemoglobin. The sapphire window was built into a thermostated block for use at 37 °C. For analyses using 16 Hz frequency, 50 µl of well mixed blood were applied to the sapphire window of the instrument and the lamp activated immediately. The instrument was calibrated using a patient pool of known haemoglobin concentration. To avoid a signal saturation effect at the lower frequency of 2 Hz, well-mixed blood was diluted four-fold with deionized water containing 2 g/l Sterox SE. The instrument was calibrated with diluted blood as mentioned above.

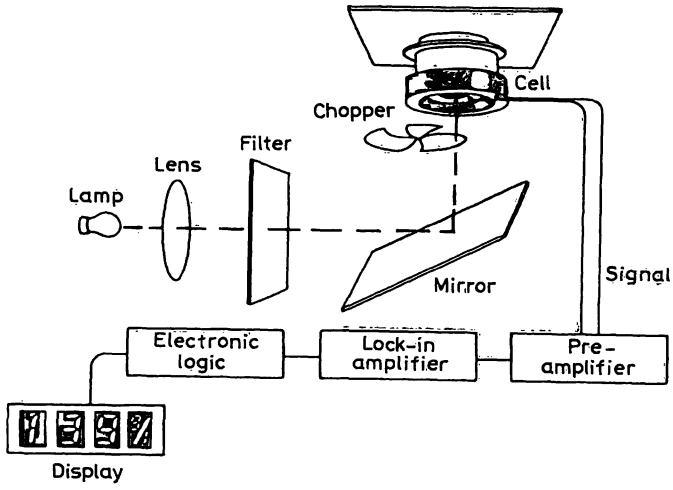

Fig. 2. Schematic representation of the optothermal spectrometer.

Within-series imprecision was calculated from consecutive analyses of three different samples. For within-day and between-day imprecision, a stock standard was prepared by mixing several samples and diluting four fold with deionzed water containing 2 g/l Sterox SE. Aliquots of 1 ml were frozen at −20 °C and two aliquots were thawed daily for analyses five times in the morning and five times in the afternoon. Within-day imprecision was also tested using patient samples (n = 20) analysed during four days. Total imprecision was calculated from the available data.

To investigate linearity, three to four samples containing approximately the same amount of haemoglobin were mixed and centrifuged. The plasma was removed and replaced by a lesser volume of 150 mmol/l NaCl. Before the analysis at 2 and 16 Hz frequencies, the samples were diluted with NaCl. Patient samples were analysed at these two frequencies and with the Coulter Counter model S-880 (Coulter Electronics, Luton, England), in which haemoglobin is analysed using the cyanide-methaemoglobin reaction (6). The haemoglobin concentrations were compared by least square regression analyses. The effect of lipaemia was investigated by adding different quantities of Intralipid (Kabi Vitrum, Stockholm, Sweden) to blood samples before analyses by optothermal spectrometry or with the Coulter S-880.

Results

The coefficient of variation (CV) in % is 100 times the quotient of the standard deviation (s) divided by the mean (x). The CVs for within-series imprecision using optothermal spectrometry at 2 and 16 Hz frequencies varied between 0.2 and 3.3% (tab. 1). The coefficients of variation for within-day imprecision

were 1.3% (16 Hz) and 4.5% (2 Hz) (tab. 2). Within-day CVs for patient samples were 3% at both frequencies. Between day CVs were approximately 5% (tab. 2). The coefficients of variation for total imprecision were 4.6% and 6.3%, for 16 and 2 Hz, respectively. The CVs for within-series, within-day, between-day and total imprecision with the Coulter S-880, using a sample containing 120 g/l haemoglobin, were 1.4, 3.0, 4.2 and 3.7%, respectively.

The signal response in optothermal spectrometry was linear up to 195 g/l haemoglobin. Method comparisons between optothermal spectrometry and the Coulter Counter were excellent at both the frequencies tested (fig. 3). The comparison of the same patient samples (n = 131) at 16 Hz (x) and 2 Hz (y) was satisfactory, giving the regression equation $y = 1.02\,x - 2.1$ (coefficient of correlation r = 0.981).

The effect of lipaemia is shown in table 3, where the results are presented as percentage recovery of haemoglobin in relation to the initial concentrations. Whereas analyses with the Coulter S-880 show a positive interference even at a relatively low degree of lipaemia, the results from optothermal spectrometry were not affected even in very lipaemic samples.

Tab. 1. Within series imprecision for haemoglobin

	16 Hz frequency			2 Hz frequency		
n	20	20	20	20	20	20
$\bar{x}$, g/l	24.9	123.4	160.1	32.3	111.8	154.7
s, g/l	0.13	2.22	2.56	1.10	0.22	0.46
CV, %	0.5	1.8	1.6	3.3	0.2	0.3

Tab. 2. Within-day and between-day imprecision for haemoglobin.

	16 Hz frequency		2 Hz frequency	
	Within-day	Between-day	Within-day	Between-day
n	20	20	20	20
$\bar{x}$, g/l	33.6	34.0	33.9	34.5
s, g/l	0.43	1.52	1.52	1.81
CV, %	1.3	4.5	4.5	5.3

Tab. 3. Recoveries (%) of haemoglobin in lipaemic samples.

Triacylglycerols (mmol/l)	Coulter S-880	Optothermal spectrometry	
		16 Hz	2 Hz
2.7	100	100	100
5.0	108	100	99
9.4	120	102	99
16.0	138	103	100
22.7	150	99	100

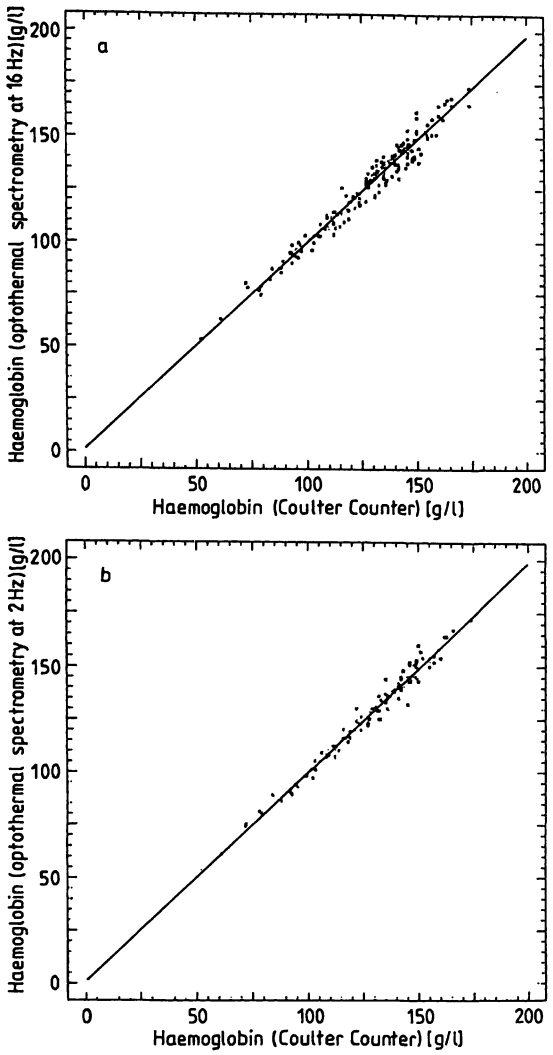

Fig. 3. Method comparisons:
a) Analyses of patient samples (n = 202) with the Coulter S-880 (x-axis) and by optothermal spectrometry at 16 Hz (y-axis). Least square regression analyses gave the equation $y = 0.98\,x + 1.1$ (r = 0.983).
b) Analyses of patient samples (n = 131) with the Coulter S-880 (x-axis) and by optothermal spectrometry at 2 Hz (y-axis). The regression equation was $y = 0.99\,x + 1.03$ (r = 0.990).

(II) Determination of the Prothrombin complex

General

The determination of the prothrombin complex is most commonly used as a "global" gauge of the functional activity of factors II, VII and X. Since these coagulation factors are vitamin K-dependent, their concentrations are affected by the administration of Coumadin (Dicumarol, Warfarin) and thus the monitoring of the prothrombin complex has particular value in following anticoagulant therapy (7–9). The test is performed by adding tissue factor to recalcified plasma, whereby prothrombin is converted to thrombin and this converts soluble fibrinogen into insoluble fibrin. The formation of the fibrin clot can be determined either visually or with the help of some

optical device. When haemoglobin is measured by optothermal spectrometry at 2 Hz frequency using a diluted sample, the light source is activated for a period of 20 seconds. If the sample was diluted with an isotonic solution and the light source activated continuously, the concentration of haemoglobin would increase gradually due to the sedimentation of the erythrocytes onto the sapphire window. When the same blood is mixed with a reagent used for the analysis of the prothrombin complex, the sedimentation of the erythrocytes either decreases or stops as soon as insoluble fibrin is produced, building a network and trapping the erythrocytes before they reach the sapphire window, thereby enabling quantitation of the coagulation time.

Materials and Methods

Blood samples collected in sodium citrate were analysed within four hours of sample collection. The contents of thrombotest reagent (batch No. 313, Nycomed & Co, Oslo, Norway) were dissolved in 11 ml of a solution containing 3.2 mmol/l $CaCl_2$ and frozen at −20 °C as 100 µl aliquots. For the analysis, an aliquot was warmed to room temperature and 20 µl of blood were added. A 40 µl portion of the mixture was transferred immediately to the sapphire window of the optothermal spectrometer (2 Hz frequency, 37 °C) and the light source activated continuously. At the same time, the chart recorder coupled to the spectrometer was started (adjustment 2 volts, chart speed 1 cm/min).

Within-series imprecision was tested at three different levels. Within-day and between-day imprecision were not tested since the coagulation factors are not stable to storage. To test linearity, a sample with a coagulation time of ca 50 seconds was diluted with 150 mmol/l NaCl and analysed in triplicate with the instrument used for routine analyses (LODE LC-61, Holland) and optothermal spectrometry. For method comparisons, patient samples were analysed with the LODE LC-61 and by optothermal spectrometry, and the results were compared by least square regression analysis.

Results

The sedimentation of erythrocytes (analysed as haemoglobin) in a sample containing 20 µl blood and 100 µl of isotonic sodium chloride is shown in figure 4. When the sodium chloride is replaced by thrombotest reagent, the sedimentation rate is altered as soon as clot formation starts (fig. 4b). The point at which this rate of sedimentation alters is measured and represents the coagulation time in seconds.

The coefficients of variation for within-series analyses varied between 4.6 and 13.9% (tab. 4). The linearity extended over the range 48 to 220 seconds. This corresponds to coagulation activities of the prothrombin complex from 45% to less than 5%. Results from the routine method and from optothermal spectrometry showed an acceptable level of agreement (fig. 5). The coagulation times were not affected by lipaemia in either method (tab. 5).

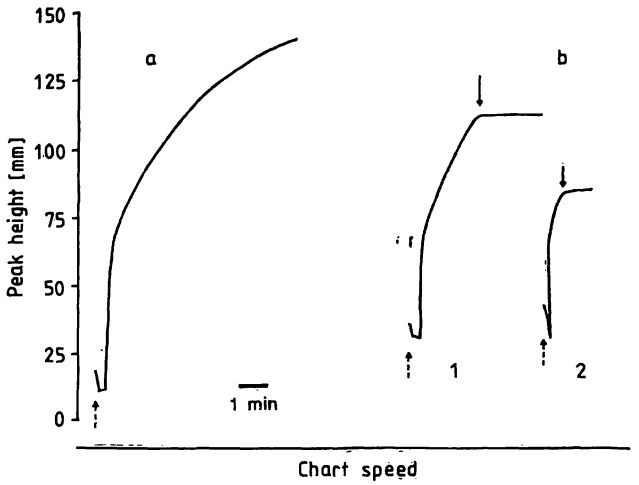

Fig. 4a. Sedimentation of erythrocytes.
Forty µl of blood diluted six-fold with 150 mmol/l NaCl were applied to the sapphire window of the optothermal spectrometer (2 Hz, 37 °C) and the lamp activated continuously. The haemoglobin in the erythrocytes was monitored on the chart recorder (speed: 1 cm/min). The start is indicated by the broken arrow.

Fig. 4b. Sedimentation of erythrocytes in the presence of thrombotest reagent.
Twenty µl of blood were mixed with 100 µl of thrombotest reagent and 40 µl applied to the sapphire window. The rest of the procedure was as in figure 4a. (1) Sample with a coagulation time of 140 s, (2) sample with a coagulation time fo 50 s. Broken arrows indicate the start, completed arrows indicate the point of coagulation.

Tab. 4. Within-series imprecision for determination of the prothrombin complex.

	Coagulation time (s)		
n	10	10	10
$\bar{x}$, seconds	51.8	96.2	167.6
s, seconds	2.39	2.70	23.26
CV, %	4.6	2.8	13.9

51.8 seconds represents a coagulation activity of 43% and 167.6 seconds an activity of 6%.

Discussion

One of the biggest advantages with optothermal spectrometry is that haemoglobin can be determined in blood without derivatization, thereby eliminating the need for any reagent. The method measures haemoglobin predominantly as oxyhaemoglobin but all derivatives having an absorbance within the wavelength region 500 to 580 nm would also be included in the determination. Identical haemoglobin results were obtained either at room temperature (ca 20 °C) or 37 °C. The choice of modulation frequency for optothermal spectrometry predetermines the method for haemoglobin analyses. Whole blood can be used directly at 16 Hz frequency. However, it is vital that the sample is thoroughly mixed and homogeneous before appli-

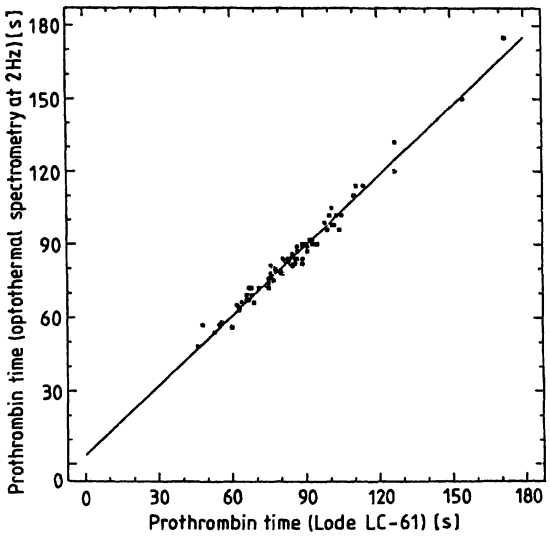

Fig. 5. Prothrombin time, method comparison.
Patient samples (n = 86) were analysed with LODE LC-61 (x-axis) and optothermal spectrometry (y-axis). Least square regression analyses gave the equation $y = 0.96x + 3.46$ ($r = 0.989$).

Tab. 5. Effect of lipaemia.

Triacylglycerols (mmol/l)	Coagulation time (s)	
	LODE	Optothermal Spectrometry
1.5	96	92
3.8	95	91
8.2	94	93
14.8	95	93
20.0	94	92

cation to the sapphire window, and that the light source is activated immediately after sample application. Duplicate readings of the same blood sample should be avoided since the second value is always increased due to the packing of the erythrocytes on the sapphire window. Since the sensitivity of the instrument is increased three-fold at 2 Hz frequency, the blood sample needs to be diluted. Even though isotonic sodium chloride can be used for dilutions, the use of deionized water containing Sterox SE is preferred, since the sample is completely haemolysed, enabling duplicate readings without encountering the problem of erythrocyte packing. Optothermal spectrometry has been tested for sample volumes varying from 10 to 100 µl without loss of sensitivity and precision.

Another advantage of optothermal spectrometry is its insensitivity to lipaemia. Since the measurement is performed in a thin layer of cells adjoining the sapphire window, it is feasible that lipids and chylomicrons, being low density particles, migrate towards the surface of the blood (away from the sapphire window) and thus have no effect on the measurements. Additionally, light scattering due to lipids which affects conventional photometry, has very little effect on optothermal spectrometry.

As shown in this study, both haemoglobin and the prothrombin complex were analysed with good precision, and the method comparisons were acceptable. A CV of 14% for prothrombin complex is not alarming since a difference in the coagulation time of 20 seconds in samples with long incubation times represents a difference in prothrombin complex of 1%.

One possible drawback with optothermal spectrometry is that samples are applied directly on the sapphire window which needs wiping after every sample. We have therefore also evaluated disposable sample carriers. Essentially, these were paper blocks the size of normal photographic slides with a hole in the middle. A thin polymer film (1.5 µm) was glued to one side producing a sample cell which could accommodate up to 200 µl sample. The sample carriers could be placed with the cell directly over the sapphire window. The polymer film in contact with the window was pretreated with silicone to permit proper adhesion. However, the polymer film dampened the signals from the spectrometer by approximately 30 and 10% for 16 and 2 Hz, respectively. This effect could be compensated mathematically for haemoglobin. No compensation was required for prothrombin complex, since this was measured as a change in the rate of sedimentation and variations in amplitude should have a minimal effect on the final outcome. Precision was acceptable at 2 Hz but poorer at 16 Hz. Method comparisons against the routine methods were acceptable.

In conclusion, optothermal spectrometry represents a simple and practical method for the analysis of haemoglobin without derivatization, either in whole blood or in a haemolysed sample. Inhibition of sedimentation is used to measure the prothrombin complex.

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Dr P. Masson
Department of Clinical Chemistry
University Hospital
S-221 85 Lund